Clonal Nature of Seborrheic Keratosis Demonstrated by Using the Polymorphism of the Human Androgen Receptor Locus as a Marker

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We evaluated the clonality of seborrheic keratoses using a polymorphism due to the random inactivation of one of two X chromosomes in females. Thirty-eight seborrheic keratoses obtained from the skin of females with polymorphism of the human androgen receptor (HUMARA) locus were examined by a fluorescent polymerase chain reaction procedure, which allowed accurate measurement of the peak intensities of each HUMARA allele. The epithelial portion of seborrheic keratosis and normal control epidermis adjacent to the seborrheic keratosis were removed by laser capture microdissection. As biopsied specimens of seborrheic keratoses contained small amounts of normal epidermis, the effect of digestion by a restriction enzyme (*Hha*I) recognizing the nonmethylated active sites was compared between seborrheic keratoses and normal control epidermis in only five seborrheic keratosis cases. Disappearance or significant reduction in intensity of one of two HUMARA alleles was observed after *Hha*I digestion in seborrheic keratoses, but not in the normal control epidermis. Although the skewing of the polymorphism was not corrected by the normal control epidermis in the remaining 33 seborrheic keratosis cases, one of two HUMARA peaks practically disappeared after *Hha*I digestion in 20 of 33 seborrheic keratosis cases. In total, 25 of 38 seborrheic keratoses were considered to be monoclonal. The histologic type of seborrheic keratoses did not affect clonality. ***Key words: clonality/human androgen receptor gene/seborrheic keratosis/X chromosome inactivation mosaicism. J Invest Dermatol 116:506–510, 2001***

Seborrheic keratosis (SK) is one of the most common benign lesions of the human epidermis (Stewart *et al*, 1978; Mackie, 1992; Ho and McLean, 1993; Kirkham, 1997). SK do not usually appear before middle age, and occur mainly on the trunk and face, but also on the extremities, with the exception of the palms and soles (Kirkham, 1997). They show a considerable variety of histologic appearances, and six types are recognized: acanthotic, hyperkeratotic, reticulated, clonal, irritated, and melanoacanthoma. Often, more than one type is found in the same lesion, and all types show hyperkeratosis, acanthosis, and papillomatosis (Kirkham, 1997).

The etiology of SK is unknown. Whether SK are hyperplastic or neoplastic has not yet been determined. If SK is a hyperplasia of the epidermis, it does not originate from a single epithelial cell and therefore is polyclonal; however, if it is a neoplasia, SK is monoclonal. As an examination of clonality appeared to promote understanding of the etiology of SK, we investigated this point. As epithelial tumors of the skin, verrucae vulgaris were first demonstrated to be monoclonal (Murray *et al*, 1971). Basal cell carcinomas are also shown to be monoclonal (Walsh *et al*, 1996, 1998). Although the former is benign and the latter malignant and, although the former is caused by a human papillomavirus, both of these epithelial lesions are now considered to be neoplasia.

To examine the clonality of human samples, random inactivation of one of two female X chromosomes is most commonly used (Vogelstein *et al*, 1985, 1987). Currently, the polymorphism of the human androgen receptor (HUMARA) locus, which is located on the X chromosome, is most frequently used as it has a highly polymorphic tandem repeat (Allen *et al*, 1992). Approximately 90% of females are heterozygous for the number of CAG trinucleotide repeats (Allen *et al*, 1992). Moreover, methylation-sensitive restriction sites of *Hpa*II and *Hha*I are within the HUMARA locus (Allen *et al*, 1992), and the base pairs amplified by polymerase chain reaction (PCR) are relatively small (Mashal *et al*, 1993). We obtained pure SK lesions using a laser capture microdissection (LCM) system (Emmert-Buck *et al*, 1996; Simone *et al*, 1998). A clonality assay using the polymorphism of the HUMARA locus indicated that more than half the SK were monoclonal in nature.

MATERIALS AND METHODS

SK samples SK lesions were biopsied from nongenital regions of 40 female patients, ranging in age from 29 to 90 y. Diagnosis was made under a microscope by examining paraffin sections stained with hematoxylin and eosin. Of the 32 specimens in which the location was specified, 12 were from the trunk, seven from the face, six from the neck, five from the upper limbs, and two from the lower limbs. Forty-four SK obtained from 40 patients were histologically classified into the following four types: acanthotic (27), hyperkeratotic (14), reticulated (two), and irritated (one).

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Abbreviations: CR, corrected ratio; LCM, laser capture microdissection; SK, seborrheic keratosis.

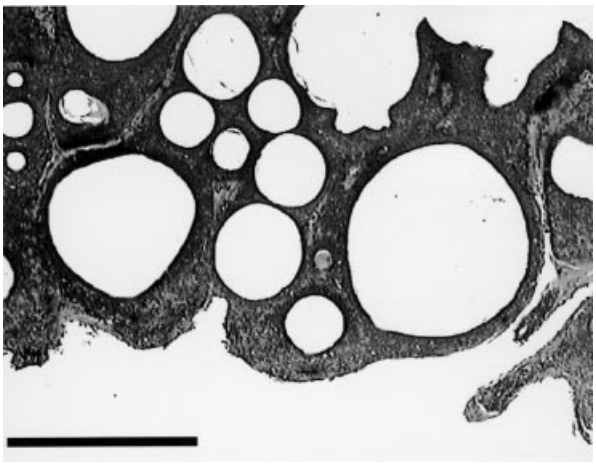

Figure 1. A SK lesion removed from patient no. 24 using the LCM system. *Scale bar*: 500 μm.

DNA extraction DNA samples were extracted from the formalin-fixed, paraffin-embedded tissues according to a previous method (Mashal *et al*, 1993). The SK lesion and normal control epidermis adjacent to the SK lesion were microdissected from 6 μm sections stained with hematoxylin and eosin using an LCM system (LM200, Arcturus Engineering, Mountain View, CA). A representative SK sample is shown in **Fig 1**. A total volume of 20 μl of PK buffer containing 1.0% proteinase K, 10 mM Tris–HCl (pH 8.0), 1 mM ethylenediamine tetraacetic acid, and 1% Tween 20 was mounted on a microdissected specimen attached to a piece of LCM Transfer Film (Arcturus Engineering). Then, a 0.5 ml micro test-tube (Eppendorf-Nethtler-Hinz-GmbH, Hamburg, Germany) was placed over it. After 16 h incubation at 37°C, proteinase K was inactivated by heating at 95°C for 10 min. The lysis mixture was centrifuged for 5 min to remove undigested tissue fragments.

Clonality assessment We used a quantitative fluorescent PCR procedure that enables accurate measurement of the peak intensities of each allele according to a previous report (Wu *et al*, 1999). The HUMARA gene includes a polymorphic [(CAG)n] repeat located at 3′ of the methylation-sensitive *Hha*I restriction-enzyme sites (Allen *et al*, 1992). The PCR assay used primers, the product of which spanned both the *Hha*I sites and the [(CAG)n] polymorphism. Variations in length of the [(CAG)n] repeats on the paternal and maternal X chromosomes yield HUMARA alleles of different lengths. Methylation of the *Hha*I sites distinguishes the active (nonmethylated) from the inactive (methylated) X chromosome. It is only the undigested inactive methylated allele that is subsequently amplified by PCR (Allen *et al*, 1992).

Each SK DNA sample or nonlesional epidermis (7.5 μl) was digested overnight at 37°C in a 2.5 μl reaction mixture containing 0.5 μl of 16 units of *Hha*I (TOYOBO, Osaka, Japan), 1 μl of concentrated (× 10) TA buffer (330 mM Tris-acetate, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM dithiothreitol), and 1 μl of concentrated (× 10) bovine serum albumin. For each case, a control sample containing only a restriction-enzyme buffer was run simultaneously. The restriction enzyme was then inactivated by heating at 95°C for 10 min.

For the PCR, 1 μl of each digested DNA sample was added to 24 μl of a PCR reaction mixture containing 2.5 μl of concentrated (× 10) PCR buffer, 1.5 μl of deoxyribonucleoside triphosphate (200 μM), 0.2 μl of primers 1A and 1B (10 pM each), 0.1 μl of *Taq* polymerase (Boehringer Mannheim GmbH, Mannheim, Germany) and 19.7 μl of deionized H_2O. The DNA samples were amplified using a sandwiched primer approach (Fujita *et al*, 1996). The first step was performed using outer primers 1A (5′-GCT GTG AAG GTT GCT GTT CCT CAT-3′) and 1B (5′-CGT CCA AGA CCT ACC GAG GAG CTT-3′). The second step was performed with inner primers 2A (5′-TCC AGA ATC TGT TCC AGA GCG TGC-3′) and 2B (5′-ATG GGC TTG GGG AGA ACC ATC CTC-3′). Primer 2A was labeled at the 5′ end with 6-carboxyfluorescein. Initial denaturation was performed for 10 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C. In the final cycle, extension at 72°C was prolonged for 10 min. The second-step PCR profile was the same as the first-step PCR. Following the second-step amplification, 5 μl of the PCR product was assessed using 2.0% agarose gel electrophoresis to confirm the amplification of the HUMARA target.

After amplification, 1 μl of the PCR products was mixed with 12 μl of a Template Suppression Reagent (Applied Biosystems, Foster City, CA) and 0.5 μl of internal size standards [GENESCAN-500 (TAMRA), Applied Biosystems). The mixture was denatured at 95°C for 2 min, and analyzed through a DNA Sequencing Polymer (Applied Biosystems) with an ABI PRISM Genetic Analyzer (Applied Biosystems), according to a previous method (George *et al*, 1997). Data were analyzed using Genescan 310 Software (Applied Biosystems).

Data interpretation Amplification of each HUMARA allele usually generated a set of multiple peaks, including one major peak and a few associated peaks of lesser intensity as described previously (Paradis *et al*, 1997). Clonality assessment was based on the major peak generated from each allele. Patients were considered heterozygous when PCR amplification of undigested DNA showed two major peaks of almost equal intensity. This suggested that maternal and paternal X chromosomes have HUMARA alleles of different molecular weights. PCR products showing a single major peak suggested that maternal and paternal X chromosomes have HUMARA alleles of the same molecular weight. Such patients were considered to be homozygous for the HUMARA gene, and thus uninformative for the analysis.

For each sample, the peak intensities of two alleles (the allele with the lower molecular weight and the allele with the higher molecular weight) were measured. A corrected ratio (CR) was assessed by dividing the ratio of the *Hha*I-digested sample (the allele with the lower molecular weight/the allele with the higher molecular weight) by the ratio of the undigested sample. The use of CR corrects for the preferential amplification of one allele that can occur if the alleles differ markedly in molecular weight (Paradis *et al*, 1997). The ratio was inverted, if necessary, to obtain a value of over 1.0.

When enough DNA was obtained from the normal epidermis adjacent to the SK lesion, the final clonality ratio for each SK was determined by dividing the CR of the lesional DNA by the CR of the nonlesional DNA. This final clonality ratio corrects for any potential skewed lyonization. We assumed that SK with a final clonality ratio of ≥ 1.5 were monoclonal, according to a previous report (Paradis *et al*, 1997).

Statistical analysis Statistical analysis was performed using Microsoft Excel (Microsoft, WA). The data are presented as mean and SEM. Differences between groups were evaluated by Student's t test.

RESULTS

SK specimens were obtained from 40 women. Sections from the SK lesions were stained with hematoxylin and eosin, and histologic types were determined. Then, the epithelial portions of the individual SK lesions were carefully removed by LCM (**Fig 1**). DNA was extracted from each sample, and the portion of the HUMARA locus containing the trinucleotide repeats was amplified by PCR. Samples derived from six of 40 patients could not be used because the polymorphism of the HUMARA locus was not detectable. The remaining 34 patients were considered to be informative. As three of 34 informative patients (12, 17, and 27) had two or three SK, 38 SK lesions were analyzed in total (**Table I**).

Histologic types, allelic sizes, and CR values of informative SK cases are shown in **Table I**. As biopsied SK specimens contained relatively small amounts of normal epidermis adjacent to the SK, CR values of the normal control epidermis were obtained in only five SK cases, and the final clonality ratio was calculated by dividing the CR of each SK by the CR of the normal control epidermis (**Table I**). The final clonality ratio of these five SK was > 1.5.

CR values were plotted against numbers of bases that were different between two HUMARA alleles of individual SK (**Fig 2**). The CR values appeared to be dependent on the number of bases, being different between alleles of lower molecular weight and those of higher molecular weight. CR values were ≥ 10 in none of the 10 SK in which 3 bp were different between the two alleles, in five of 10 SK in which 6 bp were different between the two alleles, and in 16 of 18 SK in which ≥ 9 bp were different between the two alleles (**Fig 2**). In total, CR values were ≥ 10 of 21 of the 38 SK examined.

Table I. Histologic types, size of alleles, corrected ratios and final ratios of SK cases

Patient no.	Histologic type[a]	Size of alleles (difference in bases)	CR		Final ratio (SK/normal epidermis)
			SK	Normal epidermis	
1	AC	222/225 (3)	1.5		
2	AC	219/222 (3)	1.6		
3	AC	231/234 (3)	1.7		
4	AC	225/228 (3)	2.5		
5	AC	228/231 (3)	2.9		
6	AC	216/219 (3)	3.1		
7	AC	237/240 (3)	3.8		
8	AC	228/231 (3)	4.3		
9	AC	225/228 (3)	8.1		
10	AC[b]	216/222 (6)	2.7	1.1	2.5
11	AC	225/231 (6)	3.8		
12	AC	225/231 (6)	8.7		
	AC[c]	225/231 (6)	> 10		
13	AC[c]	228/234 (6)	> 10		
14	AC[c]	222/228 (6)	> 10		
15	AC[c]	222/231 (9)	10.0		
16	AC[b]	231/243 (12)	2.5	1.0	2.5
17	AC[c]	234/246 (12)	> 10		
	AC[c]	234/246 (12)	> 10		
18	AC[b]	222/237 (15)	6.0	1.1	5.5
19	AC[c]	207/222 (15)	> 10		
20	AC[c]	222/237 (15)	> 10		
21	AC[c]	228/246 (18)	> 10		
22	AC[c]	204/222 (18)	> 10		
23	HY	231/237 (6)	2.0		
24	HY[b]	225/231 (6)	4.2	1.1	3.8
25	HY[c]	234/240 (6)	> 10		
26	HY[b,c]	228/237 (9)	> 10	1.0	> 10
27	HY[c]	228/237 (9)	> 10		
	HY[c]	228/237 (9)	> 10		
	RE[c]	228/237 (9)	> 10		
28	HY[c]	234/243 (9)	> 10		
29	HY[c]	237/246 (9)	> 10		
30	HY[c]	216/228 (12)	> 10		
31	HY[c]	225/237 (12)	> 10		
32	HY[c]	222/237 (15)	> 10		
33	RE	228/231 (3)	1.6		
34	IR[c]	228/234 (6)	> 10		

[a]AC, acanthotic; HY, hyperkeratotic; RE, reticulated; IR, irritated.
[b]Considered monoclonal because the final ratio is ≥ 1.5.
[c]Considered monoclonal because CR is ≥ 10.0.

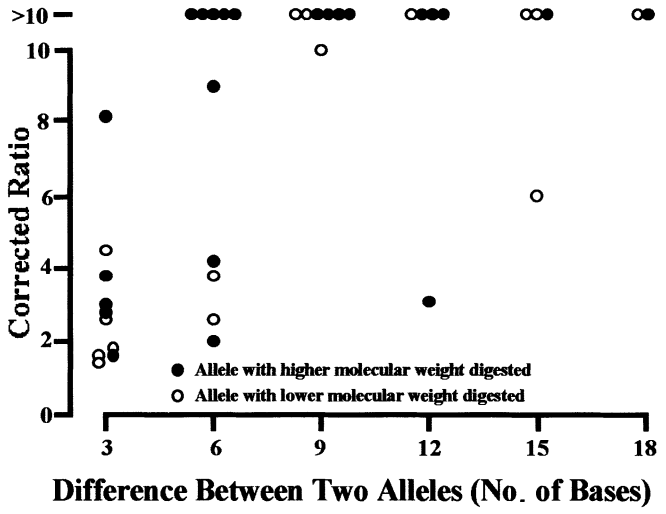

Figure 2. Interrelationship between CR values and numbers of bases that were different between two HUMARA alleles of individual SK. ●, the SK in which the HUMARA allele with higher molecular weight was digested. ○, the SK in which the HUMARA allele with lower molecular weight was digested.

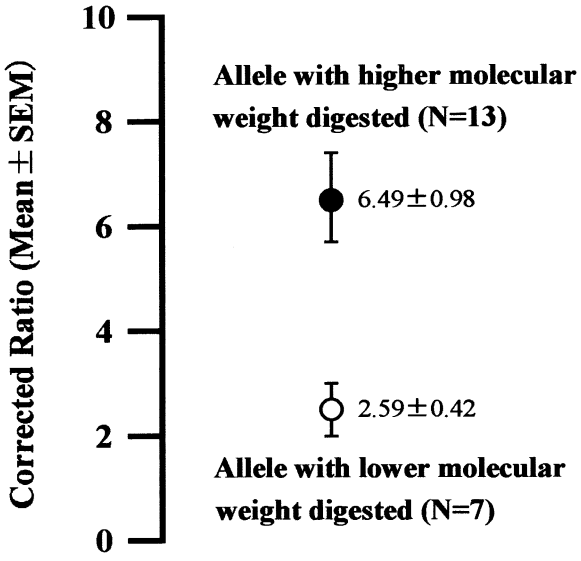

Figure 3. Effect of the size of the HUMARA allele to be digested on CR values in SK in which the two HUMARA alleles differed by 3 or 6 bp. The same results as shown **Fig 2**. The SK cases in which the allele with higher molecular weight was digested were compared with those in which the allele with a lower molecular weight was digested. Between CR values of these two groups, a significant difference was observed ($p < 0.01$ by t test).

When the number of bases that differed between the two alleles was 3 or 6, the variation in CR values was significant among individual SK (**Fig 2**). The following tendency was observed. CR values were higher when the HUMARA alleles with higher molecular weights were activated and digested than when the allele with a lower molecular weight were activated and digested (**Fig 3**). For example, compare **Fig 4**(***4A1***) (CR = 8.1) and **Fig 4**(***4A2***) (CR = 1.7), and **Fig 4**(***4B1***) (CR > 10) and **Fig 4**(***4B2***) (CR = 3.8). The number of bases that differed between the two HUMARA alleles was three in the cases shown in **Fig 4**(***4A1***, ***4A2***). Also, the number of bases that differed between the two alleles was six in the cases shown in **Fig 4**(***4B1***, ***4B2***).

DISCUSSION

In this study, we examined the clonality of SK. Biopsy specimens of SK contained only small amounts of normal epidermis. Therefore, we obtained enough normal control epidermis in only five SK cases; however, in these five SK cases, the CR values of the normal epidermis were ≤ 1.1. In other words, no significantly skewed lyonization of the normal epidermis was detected in these five cases. The final clonality ratio was calculated by dividing the CR value of each SK by that of the control normal epidermis. As a final ratio of ≥ 1.5 has been used as a criterion for monoclonality (Paradis *et al*, 1997), we considered that at least these five SK were monoclonal.

The skewing was different among various tissues from each patient. We dissected the normal epidermis using an LCM system; such skewing of the normal human epidermis has not been reported to our knowledge. To establish the normal range of CR values for the normal human epidermis, the examination of more skin samples using the present method appears to be necessary.

The slippage phenomenon observed in *Taq* amplification of trinucleotide repeats may result in minor peaks trailing the major peak at 3 bp intervals (Mutter and Boynton, 1995). This interference is insignificant when two alleles differ by 9 bp or more in size.

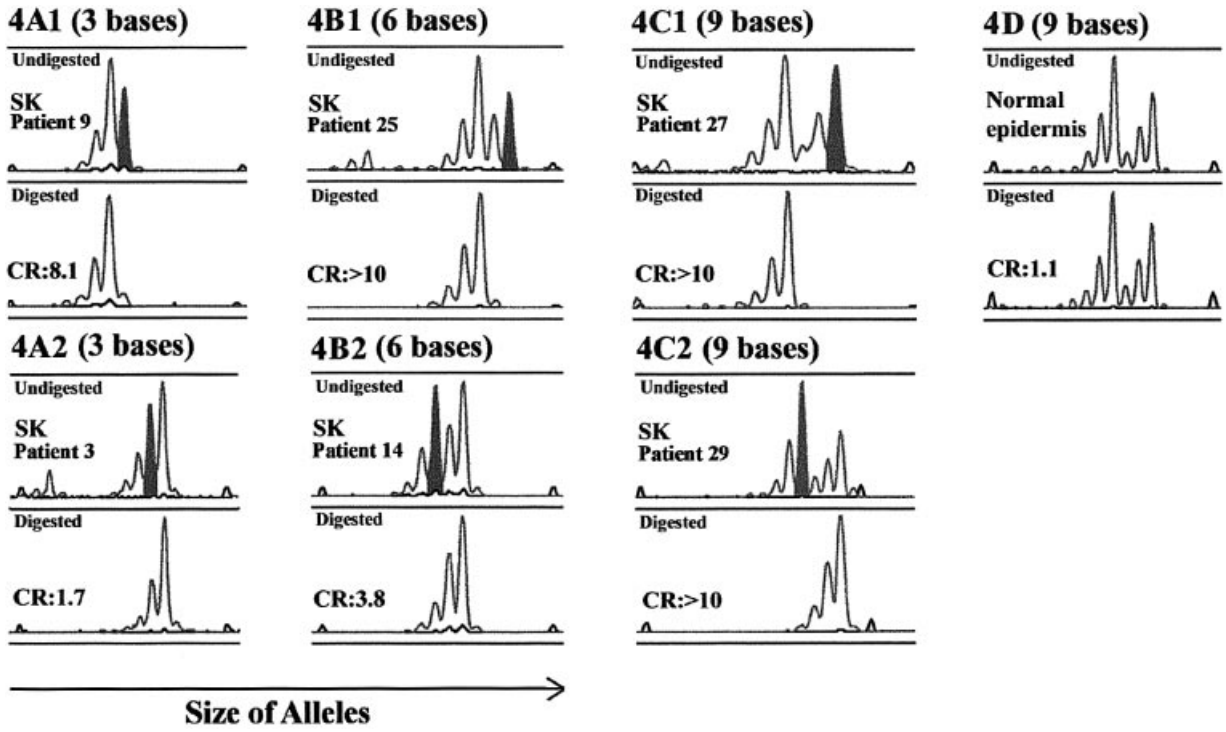

Figure 4. Comparison of clonal analysis patterns between the SK in which the HUMARA allele with higher molecular weight was digested and those in which the allele with lower molecular weight was digested. The peaks to be digested are painted out. Clonal analysis pattern of a normal control epidermis is also shown.

The two alleles differed by 9 bp or more in the 18 SK, which were examined in this study. In 16 of these 18 SK, one of two HUMARA peaks practically disappeared after digestion by *Hha*I (i.e., CR values ≥ 10.0). Therefore, at least these 16 SK were considered to be clonal.

When the two alleles differed by 6 bp, one of two HUMARA peaks also disappeared after *Hha*I digestion (CR > 10.0) in five of 10 SK cases (**Fig 2**). We concluded that these five cases were also monoclonal. Overall, five SK were considered to be monoclonal because the final ratios were > 1.5, and 21 SK were judged to be monoclonal because their CR values were ≥ 10.0. As both criteria were applicable to only one SK (patient 26), 25 of 38 SK examined were judged to be monoclonal (**Table I**). This does not imply that the remaining 13 SK were polyclonal. Although they were not determined to be monoclonal using the present method, some of them may have been monoclonal if genes on the X chromosome other than the HUMARA gene had been used as a marker of clonality.

The clonality of various skin tumors has been reported. As for epidermal tumors, verrucae vulgaris (Murray *et al*, 1971) and basal cell carcinomas (Walsh *et al*, 1996, 1998) have been shown to be monoclonal. In this study, we demonstrated that more than half of the SK were also monoclonal. Regarding mesenchymal tumors, dermatofibromas were reported to be monoclonal (Chen *et al*, 2000). Although the monoclonality of benign neurofibromas is controversial (Rey *et al*, 1987; Skuse *et al*, 1991; Daschner *et al*, 1997), neurofibrosarcomas that developed from neurofibromas were monoclonal (Friedman *et al*, 1982). With Kaposi's sarcoma, controversial results have been reported (Rabkin *et al*, 1995, 1997; Delabess *et al*, 1997; Gill *et al*, 1998). Although melanomas have been shown to be monoclonal, results regarding nevocellular neavus are controversial (Harada *et al*, 1997; Robinson *et al*, 1998). Many nontumor cells may be present within tumors. Microdissection of tissue fragments that contain tumor cells alone, and examination of clonality by a sensitive and quantitative method may be necessary to reach definite conclusions.

As mentioned previously, one artifact that may prevent accurate quantitative analysis using the present method is the slippage phenomenon observed in *Taq* amplification of trinucleotide repeats. When alleles differ by 3 or 6 bp in size, the presence of a secondary or tertiary peak may preclude the accurate analysis (Wu *et al*, 1999). We pooled the CR values of SK in which the two alleles differed by 3 and 6 bp in size. The CR values were significantly higher when alleles with higher molecular weights were digested than when alleles with lower molecular weights were digested (**Fig 3**). If alleles with higher molecular weights were digested, the intensity of the undigested alleles with lower molecular weights was unaffected (**Fig 4** ***4A1***, ***4B1***). In contrast, even if an allele with a lower molecular weight was digested, a secondary or tertiary peak of an undigested allele with higher molecular weight remained at the position where the digested peak had been situated.

Thirty-eight informative SK were histologically classified into acanthotic, hyperkeratotic, reticulated, or irritated types. SK that were demonstrated to be monoclonal were found in all histologic types. There was no correlation between histologic type and clonality (**Table I**).

In this study, we showed the monoclonal origin of more than half the SK in the skin of human females, suggesting that SK are a benign neoplasia. Although the biology of SK has not been studied in depth, it is possible that further investigation may be useful in understanding the development and progression of benign tumors in human skin.

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